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# Cisplatin activates survival signals in UM-SCC-23 squamous cell carcinoma and these signal pathways are amplified in cisplatin-resistant squamous cell carcinoma

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**Abstract.** *cis*-Diaminodichloroplatinum (II) (cisplatin) is one of the most effective anticancer drugs and is widely used for the treatment of squamous cell carcinoma (SCC). However, its efficacy is often limited due to the development of resistance. Although several factors implicated in cisplatin resistance have been identified, the resistance mechanisms in detail are not fully understood yet. In the present study, we have examined the implication of survival signaling pathways in cisplatin-resistance. Cisplatin induced activation of Ras and its downstream effector kinases, Raf/MEK/ERK in UM-SCC-23 human squamous cell carcinoma, suggesting that this anticancer drug activates survival signal pathway in addition to apoptosis signals. In cisplatin-resistant UM-SCC-23 in culture, which we have established, the protein levels of Ras, Raf-1 and MEK were drastically elevated compared to parent UM-SCC-23, and ERK and Akt signals were constitutively activated. U0126, an inhibitor for MEK and LY294002, an inhibitor for phosphatidylinositol 3-kinase (PI3K), sensitized resistant UM-SCC-23 to cisplatin-induced cell death. These results indicate that Raf/MEK/ERK and PI3K/Akt signal cascades may play a considerable role in cisplatin resistance in SCC.

## Introduction

Squamous cell carcinoma (SCC) is the largest pathogenic subpopulation among malignant tumors in head and neck region. *cis*-Diaminodichloroplatinum (II) (cisplatin) is one of the most effective anticancer drugs and is widely used for the treatment of SCC. Treatment of tumors with the DNA-damaging agents such as cisplatin elicits apoptotic death of the tumors and is initially an effective means to arrest malignancy. However, following initial success to prevent tumor progression, resistance to further cisplatin treatment often occurs (1-5). Factors implicated in cisplatin resistance include increased drug efflux, decreased drug influx, increased cellular glutathione levels, increased DNA repair, and drug tolerance (6). However, these mechanisms do not always fully account for the cisplatin resistance.

Recent studies indicated that the additional pathways, which activate anti-apoptotic and/or survival signals, may prevent the tumors from apoptosis induced by chemotherapeutic agents. In particular, the survival signals initiated by phosphatidylinositol 3-kinase (PI3K) (7) and Raf (8,9) have been characterized. The serine/threonine kinase Akt is one of the major effector molecules following PI3K activation (10). Akt is activated by phosphatidylinositol-dependent kinases (PKDs) after its recruitment to the cellular membrane by the lipid products produced by PI3K. Activated Akt can phosphorylate many downstream targets, including BAD, caspase-9, ribosomal p70 S6 kinase (p70S6K) and forkhead transcription factor. Activation of Raf is also initiated by the translocation to the cellular membrane, where it may be phosphorylated by certain kinases (11). Downstream of Raf is MEK1, a dual serine/threonine and tyrosine kinase. MEK further transmits signals to the extracellular signal-regulated kinases, ERK1 and ERK2. Activated ERK1/ERK2 can phosphorylate the p90 ribosomal S6 kinase and other CREB kinases. Therefore, PI3K/Akt and Raf/MEK/ERK signal transduction cascades can transduce signals from membrane to cell interior, in some cases to DNA transcription factors. These two signal pathways independently or synergistically block apoptosis.

In the present study, we have examined: i) whether cisplatin activates survival signaling pathways, Raf/ERK and PI3K/Akt, and ii) the changes of these pathways in cisplatin-resistant SCC. The results obtained indicate that cisplatin activates Ras and its downstream effectors Raf/MEK/ERK in SCC. In cisplatin-resistant SCC, increased expression of Ras, Raf-1 and MEK as well as constitutive activation of ERK and Akt were observed. Therefore, the survival signal

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pathways may be implicated in cisplatin-resistance and could be molecular targets for the treatment of cisplatin-resistant SCC.

## Materials and methods

*Materials.* UM-SCC-23 human head and neck squamous cell carcinoma cell line was kindly gifted from Dr Thomas E. Carey, Laboratory of Head and Neck Cancer Biology at the University of Michigan. Cisplatin was supplied from Nippon Kayaku (Tokyo, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Dulbecco's modified Eagle's medium (D-MEM) were obtained from Sigma (St. Louis, MO, USA). U0126 was from Wako (Osaka, Japan). LY294002 was from Promega (Madison, WI, USA). Phospho-ERK1/2 Pathway Sampler Kit, phosphorylated Akt, Akt and MEK1/2 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). c-Raf antibody was from Pharmingen (San Diego, CA, USA). c-H-Ras antibody was from Oncogene Research Products (Cambridge, MA, USA). Antibodies against poly(ADP-ribose) polymerase (PARP), p70S6K and phosphorylated p70S6K were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immobilon-P membrane filters were Millipore (Bedford, MA, USA). ECL Western blot detection reagents were from NEN (Boston, MA, USA). Fetal bovine serum (FBS) and penicillin/streptomycin were from ICN (Aurora, OH, USA). EZ-Detect™ Ras Activation Kit was purchased from Pierce (Rockford, IL, USA).

*Cell culture and treatment.* UM-SCC-23 cells were maintained in D-MEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (FBS/D-MEM) in a humidified atmosphere containing 5% $CO_2$ at 37°C. For experiments, cells at $10x10^5$ cells in 100-mm dishes were cultured for 24 h in FBS/D-MEM, and for another 36 h in serum-free D-MEM. Cells were then treated with cisplatin. If necessary, inhibitors were added to the culture medium 1 h prior to the addition of the anticancer drug.

*Resistant cells to anticancer drugs.* UM-SCC-23 cells were cultured (at $10x10^6$ cells in 100-mm dishes) for 24 h in FBS/D-MEM. After 24-h incubation in the serum-free medium, the cells were first treated with 0.5 μg/ml cisplatin for 24 h. After the medium was removed, cells were washed twice with phosphate-buffered saline (PBS) and were incubated in FBS/D-MEM for 5 days. This procedure was repeated 5 times until the multiplication abilities of the cells to the anticancer drug came out. Cisplatin concentrations were stepwisely increased from 0.5, 1.0, 2.0, to finally 5.0 μg/ml. After total 20 weeks, the surviving cells were designated as cisplatin-resistant cells (reSCC-23).

*Western blot analysis.* Total cellular protein extracts were used for Western blot analysis (4,12). For the preparation of cell extracts, cells were sonicated in RIPA buffer [25 mM HEPES (pH 7.4), 1% Triton-X, 150 mM NaCl, 0.1% sodium dodecylsulfate, 0.5% sodium deoxycholate, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin]. Extracted proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis, and were electrophoretically transferred onto Immobilon-P membrane. The membranes were probed with the indicated primary antibodies, and then incubated with anti-rabbit, -goat, or anti-mouse IgG horseradish peroxidase-coupled secondary antibody. Detection was performed with an ECL system. Band density was quantified by a densitometer (Densitograph, Atto, Tokyo, Japan).

*Microculture tetrazolium (MTT) assay.* For MTT assay, $1.0x10^3$ cells were plated in 96-well microplates in 100 μl FBS/D-MEM. MTT (50 μl) prepared at 5 μg/ml in PBS was added to each well. After 3-h incubation at 37°C, MTT was removed from the well, and the MTT formazan product was solubilized in 100 μl of dimethyl sulfoxide. The absorbance of the sample at 560 nm was measured using a microplate reader (Immuno Mini NJ2300, System Instruments, Tokyo, Japan). The results of the absorbance of the test wells are expressed as -fold increase in the control wells.

*Ras activation assay.* Ras activation was measured using EZ-Detect™ Ras Activation Kit. Cells ($10x10^5$ cells) were cultured in 100-mm plates containing FBS/D-MEM. Cells were quickly chilled and lysed in lysis/binding/wash buffer. Cell lysates were incubated with glutathione S-transferase (GST)-fusion protein containing Ras-binding domain (RBD) of Raf-1 (GST-Raf1-RBD) in the presence of 0.1 mM GTPγS and 6 mM $MgCl_2$. The activated Ras coupled with GST-Raf1-RBD was recovered with immobilized glutathione disc. The pulled-down active Ras was detected by Western blot analysis using anti-Ras antibody.

## Results

*Establishment of cisplatin-resistant SCC in culture.* In order to gain insight into the implication of survival signaling pathway in cisplatin-resistance, we attempted to establish cisplatin-resistant SCC in culture. When UM-SCC-23 cells were treated for 24 h with cisplatin at concentrations more than 2 μg/ml, the living cells were hardly observed after recovery culture in FBS/DMEM for 2 weeks. Therefore, cells were first treated with 0.5 μg/ml cisplatin and its concentrations were stepwisely increased from 0.5, 1.0, 2.0, to finally 5.0 μg/ml. After 20 weeks, the surviving cells were designated as cisplatin-resistant cells (reSCC-23). There were no significant differences in the morphology and proliferation rate in a regular culture medium between parent UM-SCC-23 and reSCC-23.

Fig. 1A shows the changes of cell numbers, as assessed by MTT assay, treated with various concentrations of cisplatin. Cell numbers were concentration-dependently decreased by cisplatin with $IC_{50}$ value being 1.0 μg/ml for UM-SCC-23 and 5.2 μg/ml for reSCC-23. The decrease of cells was due to apoptotic cell death since degradation of PARP, a substrate of caspase-3, was evident (Fig. 1B).

*Cisplatin-induced activation of Raf/MEK/ERK signal pathway in SCC.* Western blot analysis revealed that phosphorylated forms of Raf-1, MEK and ERK increased in response to 1 μg/ml cisplatin in UM-SCC-23 (Fig. 2), suggesting activation of

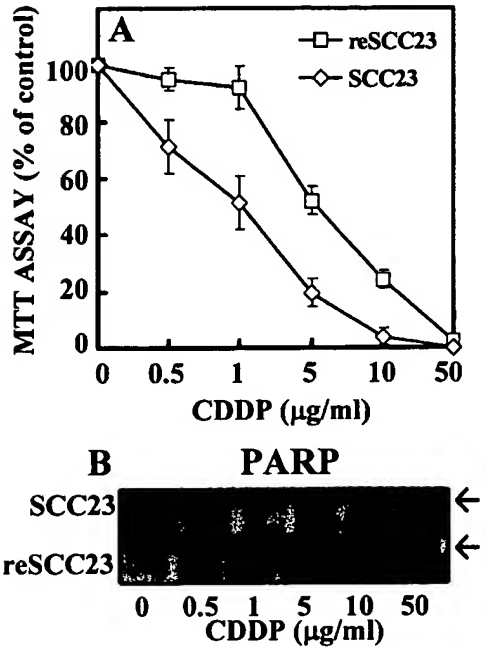


Figure 1. Cytotoxicity of cisplatin in UM-SCC-23 and reSCC-23 cells. A, Cell viability was assessed by MTT assay. Cells were treated with various concentrations of cisplatin for 24 h. The results were expressed as percentages of the untreated controls. Data are means ± SD from three independent experiments, each performed in triplicate. B, Cleavage of the caspase substrate PARP. UM-SCC-23 (SCC23) and reSCC-23 (reSCC23) cells were treated with 1 μg/ml cisplatin (CDDP) for 24 h. PARP cleavage was assessed by Western blot analysis using anti-PARP antibody. The arrows indicate the position of uncleaved PARP. The results shown are representative of at least three independent experiments.

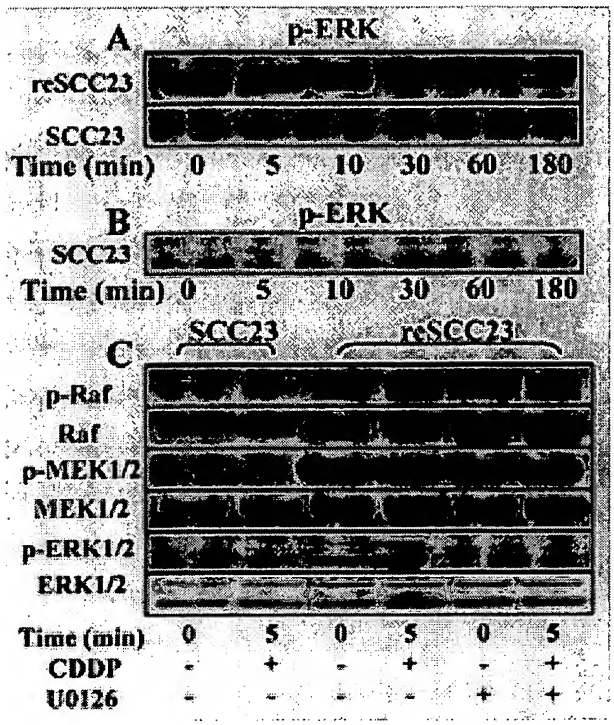


Figure 2. Cisplatin activates the c-Raf/MEK/ERK signaling pathway in UM-SCC-23 and reSCC-23 cells. A and B, Time-dependent cisplatin-induced phosphorylation of ERK. UM-SCC-23 (SCC23) and reSCC-23 (reSCC23) cells were exposed to 1 μg/ml cisplatin (CDDP) and incubated for the indicated periods of time. B, Lower panel in A was exposed to the film three-times longer. C, Cells were treated with 1 μg/ml cisplatin (CDDP) for 5 min in the absence or presence of 10 μM U0126. Cellular proteins were subjected to Western blot analysis with the indicated antibodies. The results shown are representative of at least two independent experiments.

Raf/MEK/ERK cascade. Moreover, cisplatin induced activation of Ras (Fig. 3). These results indicate that cisplatin activates survival signals in addition to apoptotic signal pathways as previously described (1-5). However, clear data showing cisplatin-induced activation of Akt and p70S6K were not obtained in spite of repeated experiments (Fig. 4).

*Amplification of Raf/MEK/ERK and PI3K/Akt signals in cisplatin-resistant SCC, reSCC-23.* Constitutive activation of ERK (Fig. 2) and Akt (Fig. 4) was evident in reSCC-23, although the protein levels of these kinases were almost the same as those in parent UM-SCC-23. On the other hand, the protein levels of Raf and MEK, upstream activators of ERK, were drastically increased compared with UM-SCC-23. In addition, upregulated Ras was constitutively activated in reSCC-23. Activated Ras is known to utilize PI3K/Akt survival signal pathway as well as Raf/MEK/ERK (13,14). Consistent with this notion, Akt was constitutively activated in reSCC-23.

U0126, a MEK inhibitor (13,15), almost abolished constitutive activation of ERK in reSCC-23, although it had little effects on constitutive phosphorylation of Raf and MEK. Interestingly, this inhibitor significantly blocked constitutive phosphorylation of Akt. Constitutive phosphorylation of Akt was nearly abolished by LY294002, a

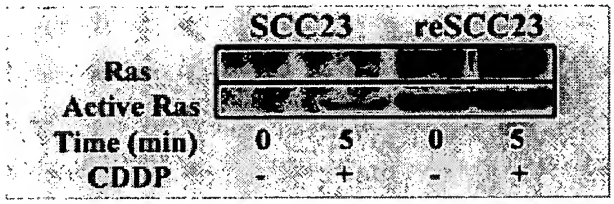


Figure 3. Ras activation in UM-SCC-23 and reSCC-23 cells. UM-SCC-23 (SCC23) and reSCC-23 (reSCC23) cells were treated with 1 μg/ml cisplatin (CDDP) for 5 min. Ras protein levels in total cellular extracts were determined by Western blot analysis using an anti-Ras antibody. The amount of activated Ras was quantified by pull-down assay using GST-Raf1-RBD fusion protein. The pulled-down active Ras with glutathione disc was detected by Western blotting using anti-Ras antibody. The results shown are representative of at least two independent experiments.

PI3K inhibitor (7,14,15), suggesting signal relay from PI3K to Akt in reSCC-23.

*Sensitization of resistant SCC to cisplatin-induced cell death by inhibitors of MEK and PI3K.* The above results clearly indicate that growth/survival signals, Raf/MEK/ERK and PI3K/Akt, are amplified in reSCC-23. We therefore tested for requirement of these signals in cisplatin-resistance using

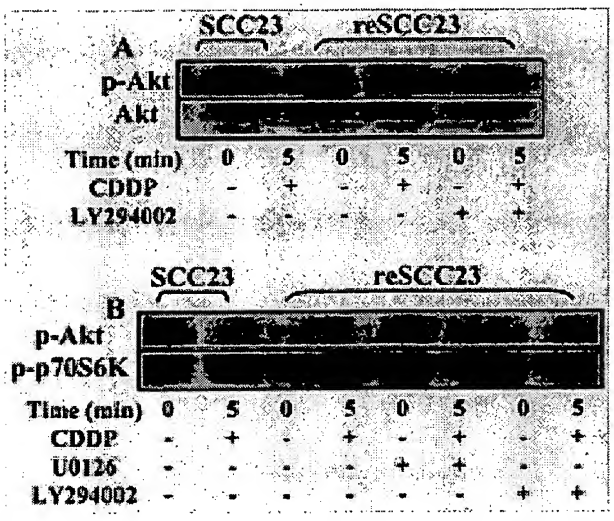


Figure 4. Western blot analysis of PI3K/Akt signaling pathway and phosphorylation of p70S6K. UM-SCC-23 (SCC23) and reSCC-23 (reSCC23) cells were treated with 1 μg/ml cisplatin (CDDP) for 5 min in the absence or presence of PI3K inhibitor LY294002 (10 μM) or MEK inhibitor U0126 (10 μM). The results shown are representative of at least two independent experiments.

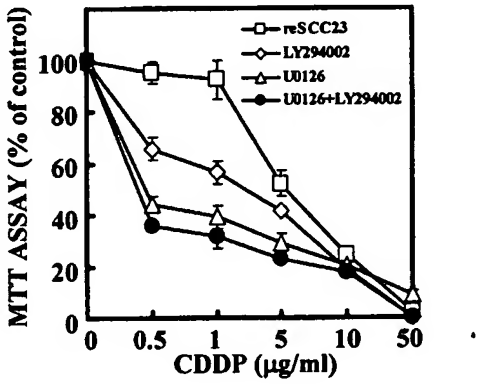


Figure 5. Effects of the PI3K inhibitor LY294002 and MEK1/2 inhibitor U0126 on cisplatin-induced cell death. reSCC-23 (reSCC23) cells were pretreated with 10 μM LY294002, 10 μM U0126 or their combinations for 1 h and then exposed to the indicated concentrations of cisplatin (CDDP) for 24 h. Cell viability was assessed by MTT assay. Data are means ± SD from three independent experiments, each performed in triplicate.

the inhibitors, U0126 for MEK and LY294002 for PI3K. As shown in Fig. 5, 10 μM of U0126 at a concentration, which almost abolished ERK activation, sensitized reSCC-23 to cisplatin-induced cell death. LY294002 showed similar sensitization effect. However, their synergistic effects were not observed. The sensitization effect obtained in combination with U0126 and LY294002 was almost the same as that of U0126 alone.

## Discussion

The implication of proliferation/survival signaling cascades in cisplatin resistance was investigated in the present study. We established a cisplatin-resistant SCC line (reSCC-23) in culture. In reSCC-23, the expression of Ras is remarkably increased and two Ras effector pathways, Raf/MEK/ERK and PI3K/Akt pathways are constitutively activated. Inhibitors of these two pathways, LY294002 for PI3K and U0126 for MEK, sensitized reSCC-23 to cisplatin-induced cell death. These results indicate that survival signaling pathways may play a considerable role in resistance to cisplatin.

PI3K activity is known to be required for growth factor-dependent survival of a wide variety of culture cells (7,13,16,17). Cells deprived of growth factor support become resistant to apoptosis, when they are transfected with constitutively active PI3K (18) or active Ras mutants (14) which bind to and activate PI3K. Among targets downstream of PI3K, Akt is sufficient to block apoptosis induced by a variety of death stimuli (7,10). Therefore, Akt is regarded as a mediator of the PI3K survival signal. Akt has been shown to exert antiapoptotic role via phosphorylation of proapoptotic proteins BAD and caspase-9, and also probably through activation of transcription factors. The results obtained in the present study with LY294002 implicate PI3K/Akt signal cascade in cisplatin resistance of SCC.

The mitogenic signal cascade composed of Raf/MEK/ERK has also recently been implicated in the apoptosis suppression. Analysis of apoptotic suppression by Raf-1 first demonstrated the requirement for mitochondrial translocation of the kinase (8). Mitochondrially active Raf-1 is unable to activate ERK1/2 but suppresses cell death by inactivating the pro-apoptotic Bcl-2 family member BAD. However, genetic and biochemical studies have also suggested a role for MEK/ERK in apoptosis suppression. For example, expression of a constitutively active form of ERK2 as well as oncogenic MEK protects NIH3T3 fibroblasts against doxorubicin-induced cell death (13). Activation of Raf-1/MEK/ERK cascade by Ras mutant reduces neuronal cell death induced by cytosine arabinoside (17). In the present study, U0126, a MEK inhibitor, sensitized reSCC-23 to cisplatin-induced cell death, clearly indicating the involvement of Raf/MEK/REK cascade in cisplatin resistance, not Raf-1 alone.

Accumulating evidence indicates various levels of crosstalk between PI3K/Akt and Raf/MEK ERK pathways. In some cases (19-22), Akt can phosphorylate Raf and result in its inactivation. For example, in MCF-7 human breast cancer cells (20), high Raf activity induces growth arrest, whereas high PI3K/Akt activity correlates with cell growth. The inactivation of the crosstalk between the two pathways switched the cellular responses from proliferation to cell cycle arrest. In neuronal cells, both pathways act independently. There is no overlap in the processes that are targeted by each pathway (16,17). In rat sympathetic neurons (17), only PI3K/Akt prevents death due to nerve growth factor deprivation, whereas Raf/MEK/ERK solely promotes neuro-protection against cytosine arabinoside. Similarly, in cortical neurons (16), ERK and Akt signaling pathways differentially mediate neuroprotection against camptothecin, an inhibitor of DNA topoisomerase-1, and neurotrophic factor withdrawal, respectively. On the other hand, Raf and Akt show positive interactions and synergize for survival in a variety types of cells (13-15,23-25). In some scenarios, Raf activity is necessary for the antiapoptotic effect of PI3K/Akt pathway

(23). On the contrary, PI3K/Akt is required for Raf-induced survival in several types of cells (13,15). In the present study, it is clear that both survival pathways are implicated in cisplatin resistance. However, U0126 and LY294002 did not show synergistic effects on sensitization of reSCC-23. U0126, at a concentration, which almost abolished constitutive ERK activation, blocked constitutive activation of Akt in reSCC-23. Therefore, it is reasonable to speculate that PI3K/Akt activity is positively controlled by Raf/MEK/ERK signal, although the clarification of signal relay in detail awaits further investigations.

Our studies indicate that cisplatin activates the Raf/MEK/ERK cascade in addition to apoptotic signals in SCC and that Raf/MEK/ERK and PI3K/Akt survival signals are constitutively activated in cisplatin-resistant SCC. Since inhibitors for MEK and PI3K sensitized the cisplatin-resistant cells to cisplatin challenge, the molecules implicated in survival signals could be targets for the treatment of cisplatin-resistant SCC.

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